Research Article

Encapsulation Strategies and Functional Quality of Symbiotic *Bifidobacterium bifidum* FNCC0491 and Inulin

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Abstract | Symbiotics, a combination of probiotics and prebiotics, play an important role in improving the digestive health of ruminants; however, bacterial viability often decreases during processing. This study aimed to evaluate the effectiveness of three encapsulation methods, which are spray drying (P1), freeze drying (P2) and cell immobilization (P3), in preserving the viability and physical quality of a symbiotic product consisting of *Bifidobacterium bifidum* as a probiotic and inulin as a prebiotic. Each method was combined with different coating materials: maltodextrin (M1), alginate (M2), and xanthan gum (M3). The parameters observed included SEM, FTIR, color, aroma, texture, dry matter and organic matter degradation and digestibility (DMD, OMD, IVDMD, IVOMD) through an *in vitro* rumen gastrointestinal simulation, and bacterial viability. The results showed that the encapsulation method and coating material had significant effects on all parameters. The best results from the P3M2 treatment were mainly due to the protective gel network formed by alginate, which improved the integrity of the microcapsules and maintained bacterial viability after encapsulation, which was regrown after 24 hours. In the cell immobilization process, alginate reacts with calcium chloride ($CaCl_2$) to form a stable calcium-alginate gel matrix without exposure to high temperatures, thereby reducing thermal damage to bacterial cells. SEM and FTIR analyses confirmed that alginate produces dense and stable microcapsules with strong molecular interactions, enabling P3M2 to maintain the highest bacterial viability among all treatments. These findings highlight that the encapsulation method of cell immobilization with alginate matrix is capable of producing microcapsules with a stable gel structure, maintaining the highest bacterial viability, and showing protected to degradation and rumen digestibility, and optimal potential for post-rumen delivery. This method is a promising approach for the development of robust, high-efficacy symbiotic feed additives.

Keywords | *Bifidobacterium bifidum*, Encapsulation, Inulin, Spray drying, Symbiotic

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INTRODUCTION

Ruminants have a unique digestive system that relies on rumen fermentation to digest crude fiber, while post-rumen tracts such as the abomasum, small intestine, and colon play a role in nutrient absorption. The balance of microbiota in these tracts is critical to maintaining digestive health and efficiency. Microbiota disruption, such

as pathogen proliferation, can reduce digestive efficiency and animal health. The addition of probiotics to feed, such as PE goats, has been shown to increase feed protein utilization as well as feed consumption (Raguati, 2016), as probiotics can stimulate metabolism and support nutrient absorption (Dubois *et al*., 2013).

One potential probiotic is *Bifidobacterium bifidum*, which is able to inhibit the growth of pathogens such as *E. coli*, *Salmonella*, and *Clostridium* through the production of lactic and acetic acids that lower gut pH (Anindita and Anwar, 2021). These probiotics also strengthen the immune system, improve gut integrity, and support nutrient absorption (Yatim, 2019). In ruminants, their use is focused on the post-rumen tract because neutral rumen conditions (pH 6.8-7.0) are less favorable for their growth (Mirahsanti *et al*., 2022), while the post-rumen section provides acidic conditions and suitable substrates for optimal growth. There is scientific evidence regarding the ability of *B. bifidum* to survive and colonize effectively in the ruminant post-rumen tract is still limited. Most existing studies only report the survival of *B. bifidum* in non-ruminant mammalian models, while applications in ruminants are still based on assumptions regarding pH sensitivity and its potential physiological adaptation to lower intestinal conditions.

A major constraint in the use of probiotics is their low viability during storage and as they pass through the digestive tract (Sakoui *et al*., 2022). Instability at room temperature, such as exposure to oxygen, humidity, and high temperatures during processing, can reduce the effectiveness of probiotics. Acidic and enzymatic conditions in the digestive tract also reduce the viability of probiotics before they reach their target location, requiring encapsulation technology to protect probiotic cells from harmful environments and extend their shelf life (Mislah *et al*., 2018). Encapsulation works by trapping sensitive microorganisms in a protective layer, improving stability, controlling the release of active compounds, and minimizing nutrient loss (Kent and Doherty, 2014; Champagne and Fustier, 2007). However, the effectiveness of existing encapsulation methods is still limited, so exploration of new matrices is needed (Mislah *et al*., 2018).

The addition of prebiotics in encapsulation can enhance the protection of probiotics. Prebiotics are substrates that microbes utilize to provide health benefits (Bindels *et al*., 2015). Symbiotics, a combination of probiotics and prebiotics, have been shown to improve microbiota health without adverse effects (Pandey *et al*., 2015). Inulin, one of the commonly used prebiotics, is a polymer of fructose that can be fermented by gut microflora (Chi *et al*., 2011) and shown to increase the abundance of *Bifidobacterium* (Rubin *et al*., 2022; Gallina and Barbosa, 2022). Inulin has the potential to be an encapsulant matrix for *Bifidobacterium bifidum* because it can provide synergistic effects, protect probiotics until they reach the post-rumen, and extend shelf life.

To date, there is no encapsulation method that can consistently maintain probiotic viability during storage and after passing through the digestive tract, especially for pH-sensitive species such as *Bifidobacterium bifidum*. This gap highlights the need for comparative evaluation of methods and coating materials to determine the most effective encapsulation technique that not only ensures high viability but also allows for industrial-scale application. Commonly used techniques such as spray drying, freeze drying, and cell immobilization (Keder and Hashish, 2020; Buljeta *et al*., 2022; Aghajani et al., 2020) need to be further studied to produce effective symbiotic products for improving livestock health and productivity. Encapsulation methods offer their own advantages and disadvantages in producing encapsulated products. Therefore, this study aims to evaluate various encapsulation methods applied to *Bifidobacterium bifidum* based symbiotic products with inulin and assess the physical quality, stability, and effectiveness of the resulting products.

MATERIALS AND METHODS

The experimental procedures in this study were carried out in several stages, including conditioning of culture bacteria, preparation of pre-culture and culture medium, matrix preparation, and encapsulation using spray drying, freeze drying, and cell immobilization. Furthermore, several analytical tests were performed, including sensory analysis, scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), total plate count (TPC), and in vitro degradation and digestibility. The overall workflow of the methods is summarized in the flow diagram presented in Figure 1.

Conditioning of culture bacteria

First, *Bifidobacterium bifidum* FNCC 0491 (sourced from the Food and Nutrition Culture Collection, Gadjah Mada University, Yogyakarta) cultures that had been inoculated in de Man, Rogosa, and Sharpe (MRS Broth) media were left at 37˚C for 24 hours to obtain fresh cultures. After that, the culture medium containing the inoculum was put into an anaerobic jar. Before closing the jar, a flushing process was carried out by flowing CO_2 gas into the container. CO_2 gas is obtained from a gas cylinder connected to the jar through a hose. This process aims to replace oxygen in the jar and create anaerobic conditions necessary for the growth of *Bifidobacterium bifidum*. After flushing is complete, the anaerobic jar is sealed and re-incubated at

37°C for 48 hours. During this incubation period, bacterial growth can be monitored through changes in the culture medium, such as colony growth or color changes, which indicate successful anaerobic conditioning.

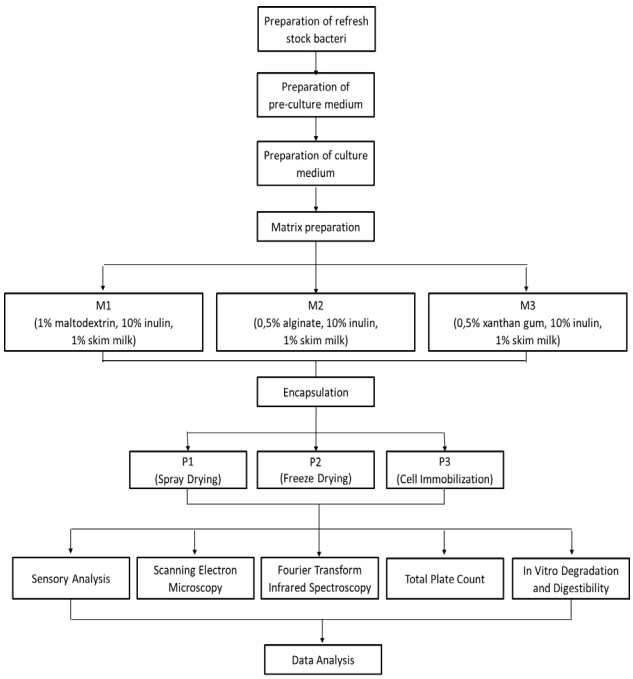

Figure 1: Flowchart of the research method.

Preparation of *Bifidobacterium bifidum* pre-culture medium

Bifidobacterium bifidum cultures for the encapsulation process were first grown in liquid MRS-Broth media (Merck, Cat. No. 1.10661.0500). This medium was made by dissolving 5.2 g of MRS-Broth powder and 1 g of peptone into an erlenmeyer with a capacity of 250 ml, then 100 ml of distilled water was added. The inoculation process was carried out by adding 1 ml of *Bifidobacterium bifidum* bacterial suspension using a micropipette in a laminar airflow and near a Bunsen flame to maintain aseptic conditions. After inoculation, the culture was incubated for 24 hours in an incubator.

Preparation of culture medium

The process of making culture media begins with dissolving 20.8 g of MRS-Broth powder in 400 ml of distilled water. This mixture was put into an erlenmeyer with a capacity of 500 ml so that the media had enough space during the sterilization process. Sterilization was carried out using an autoclave at 121°C for 15 minutes to ensure the media was free from microbial contamination. After the sterilization process is complete and the media is cool enough, the pre-culture media that has been incubated previously is mixed with the new culture media. The mixing process is done in laminar airflow to maintain aseptic conditions. The culture was then incubated in a shaker water bath for 24 hours to provide optimal conditions for bacterial growth.

Matrix preparation

The matrix preparation process began by preparing 500 ml of distilled water in a large Erlenmeyer flask as the base solution. In this study, three different coating materials were used for each treatment, namely maltodextrin (M1), alginate (M2), and xanthan gum (M3). For each respective treatment, 1% maltodextrin (M1), 0.5% alginate (M2), or 0.5% xanthan gum (M3) was added to the solution, along with 10% inulin and 1% skim milk as supporting components. The mixture was stirred using a magnetic stirrer until all ingredients were completely dissolved to form a homogeneous matrix solution. After mixing, each matrix solution was sterilized separately in an autoclave at 121°C for 15 minutes to ensure all components were free from microorganisms and contamination.

Spray drying

Before starting the drying process, 500 ml of bacterial biomass suspension was mixed with 500 ml of sterilized encapsulant matrix solution. Mixing was done using a magnetic stirrer to ensure that the two components were evenly mixed, resulting in a homogeneous mixture. After complete mixing, the mixture of bacterial biomass and matrix was put into a spray dryer. The spray dryer and compressor are activated until the pressure reaches 60 psi. The parameters are set at a flow rate of 4.2 mL/minute (average of 500 mL/2 hours), with a fan speed of 45%, the inlet temperature is maintained at 120–130°C, and the outlet temperature is controlled at 60–80°C. This procedure was carried out to optimize the encapsulation of bacterial biomass in the matrix through efficient and controlled drying. After the drying process was complete, the encapsulation results were collected from the spray dryer collection chamber (Hermayani *et al*. 2001).

Freeze drying

Bacterial biomass suspension was mixed into the matrix solution in a sterile container and homogenized using a magnetic stirrer until evenly mixed. The mixture is then poured into trays or small freeze-proof vials and frozen in a freezer at -20°C to -80°C for at least 24 hours until the solution is completely frozen. After freezing, the trays or vials are put into a freeze dryer, where the drying temperature is set at about -40°C to -50°C with low pressure (about 0.1-0.2 mbar). The freeze-drying process lasts for 72 hours or until all the water is removed and a porous and stable dry product is produced. After the process is complete, the dried encapsulated product is taken from the device and stored in a closed container (Carvalho *et al*., 2004).

Cell Immobilization

A well-grown bacterial culture is added to the matrix solution and blended to produce a homogeneous mixture. The mixture was then dripped into a sterile 0.1 M $CaCl_2$

solution from a height of 5 cm. This mixture was stirred at 150-200 rpm using a magnetic stirrer. The gel hardening process lasted for 30 minutes. Afterwards, the beads were filtered and rinsed using a sterile 0.85% NaCl solution. The wet beads obtained were then placed in a sterile container or bottle. After the process was completed, the resulting encapsulated particles were dried using an oven at 50°C for 24 hours to remove any remaining moisture. The dried encapsulated results were then weighed to record the total weight and yield produced, and stored in an airtight container to maintain stability and viability of the bacteria (Krasaekoopt *et al*., 2003).

Analytical procedures

Sensory analysis

The sensory test involved 25 semi-trained panelists who had knowledge or expertise in recognizing symbiotic characteristics according to the modified SNI (2006) standard. The panellists were selected based on the criteria of having no impaired sense of smell, not being color blind, and being able to provide consistent assessments in accordance with sensory panellist selection guidelines (Bhuker and Maurya, 2024). Panellists' perceptions of each sample were used to evaluate the attributes of color, aroma, and texture, based on their preferences for the tested samples. Each sample was coded with a treatment number and presented randomly. Assessments were recorded using a prepared form with a 1–4 scale for attribute intensity. The results of the panellists' evaluations were then used to determine the overall acceptance level of each sample.

Scanning electron microscopy (SEM)

The encapsulated symbiotic product was placed on a round brass piece (stub) with a diameter of 1 cm. This piece was fitted with double-sided tape to ensure the powder remained stable in position. After that, the powder is made conductive through an electroplating process, where a thin layer of platinum is applied using a special beam. This coating process lasts for 30 seconds at a pressure below 2 Pa and with an electron voltage of 10 kV. After coating, the powder was observed at 500x, 2500x, and 5000x magnification to obtain a more detailed visualization of its structure and morphology (Fujita *et al*., 1971).

Fourier transform infrared spectroscopy (FTIR)

A total of 0.5 grams of symbiotic encapsulated product sample was placed in the device to identify the functional groups contained in the sample. FTIR spectra were obtained using two Perkin Elmer Spectrum FTIR-UATR instruments. The symbiotic product powder from each encapsulation method was dispersed in KBr matrix (100 mg) and compressed to pellet form for observation. Spectra were taken with 32 scans in the range of 4000 to 400 cm^{-1}, with a resolution of 4 cm^{-1} (Holme and Peck, 1993).

Viability of encapsulated symbiotics determined by total plate count

The initial bacterial population prior to encapsulation was determined using the Total Plate Count (TPC) method to establish the baseline viability of *Bifidobacterium bifidum*. Briefly, 1 mL of the cultured bacterial suspension in MRS broth was serially diluted in 9 mL of sterile distilled water until the desired dilution was achieved. Each dilution was plated on Plate Count Agar (PCA) and incubated at 37 °C for 24–48 hours. The resulting colony counts were used as a reference to calculate the survival rate after encapsulation. For the symbiotic encapsulated samples, approximately 1 g of the product was homogenized in 9 mL of sterile MRS Broth to obtain the first dilution (10^{-1}), providing a suitable environment to maintain bacterial viability. Subsequently, serial dilutions were prepared by transferring 1 mL of the previous dilution into 9 mL of sterile distilled water until the 12th dilution or the desired dilution level. From each dilution, 0.1 mL was inoculated onto PCA plates (Millipore, Cat. No. 1.05463.0500) and evenly spread using a sterile glass rod. The plates were incubated at 37 °C for 24–48 hours. After incubation, plates containing 25–250 colonies were selected for counting (Woraharn *et al*., 2010).

The total viable cell count was calculated using the following formula:

$$\text{TPC (CFU/g)} = \frac{\text{Number of colonies counted}}{\text{Dilution factor x Volume plated (mL)}}$$

The viability percentage of encapsulated bacteria was calculated as:

$$\text{Viability (\%)} = \frac{\text{Total viable cell count after treatment}}{\text{Initial viable cell count before treatment}} \times 100$$

In vitro degradation and digestibility

The ration used in the *in vitro* test was prepared based on the formulation of the farm in Pangalengan which was adjusted to the NRC (2001) for dairy cows. Table 1 presents the composition of feed ingredients and nutrient content of the rations used. A two-stage in vitro method based on Tilley and Terry (1963) was used to simulate the digestive process in ruminant animals, namely rumen fermentation followed by enzymatic digestion. Dry matter degradation (DMD) and organic matter degradation (OMD) after rumen incubation, as well as dry matter digestibility (IVDMD) and organic matter digestibility (IVOMD) after pepsin HCl enzyme treatment were assessed.

Table 1: Ration composition and nutrient content.

Feed ingredients	Ration composition (%)
Pennisetum purpureum	60,00
Corn	7,15
Soybean meal	4,40
Premium concentrate	28,30
DCP	0,15
Nutriens	**Nutrient content (%)**
Ash	9,84
Crude Protein (CP)	13,72
Crude Fiber (CF)	21,65
Ether Extract (EE)	3,71
Nitrogen-Free Extract (NFE)	51,08
Total Digestible Nutrient (TDN)	63,30

Source: Results of ration formulation calculations Laboratory. BETN = 100 – (Ash+CP+ CF+EE).

Feed samples were dried in a 60 °C oven for 48 hours, finely ground to pass a 1 mm sieve. A total of 0.5 grams of sample was weighed and put into the fermenter tube. Fresh rumen fluid was collected from fistula dairy cows using a thermos that had been warmed and filtered through a cloth. The rumen fluid was mixed with McDougall buffer solution in a ratio of 1:4 (v/v). A total of 40 ml of buffer and 10 ml of rumen fluid were added to the tube containing the sample. The tube was supplied with CO_2 gas to maintain anaerobic conditions and then sealed with rubber and incubated in a 39°C water bath for 48 hours, while being shaken periodically. This stage simulates fermentation in the rumen. Afterwards, pepsin HCl was added to some tubes to simulate post-rumen digestion (hydrolytic digestion). The sediment obtained from the centrifugation results was taken and 50 ml of HCl pepsin solution was added with a pepsin to HCl ratio of 1:3. Next, the sample was incubated for 48 hours in a water bath.

Measurement of dry matter and organic matter degradation (DMD and OMD)

After incubation for 48 hours, the tubes were removed from the water bath and immediately centrifuged at 5,000 rpm for 10 minutes to separate the solid residue from the fermentation liquid. The supernatant was carefully discarded, while the solid precipitate (residue) was collected for further analysis. The residue was then dried in an oven at 105°C for 24 hours. The sample was cooled in a desiccator and then weighed to obtain the dry weight of the residue. The residue was then dried in a furnace at 600°C for 4 hours to obtain the ash weight. Degradation calculations were carried out as follows:

$$DMD = \frac{\text{dry metter of sample} - \text{dry metter of residue}}{\text{dry metter of sample}} \times 100$$

$$OMD = \frac{\text{organic metter of sample} - (\text{organic residue weight} - \text{ash weight})}{\text{organic metter of sample}} \times 100$$

Measurement of Dry matter and organic matter digestibility (IVDMD and IVOMD)

The incubated sample was then filtered using filter paper (Whatman No. 1) with the help of a Buchner funnel and vacuum pump. The residue was washed using warm water until clean from the fermentation liquid. The vacuumed samples were stored in a porcelain cup, then dried in an oven at 105°C for 24 hours. Afterwards, the samples were cooled in a desiccator and weighed to obtain the final weight to determine the moisture content. Next, the samples were ignited in a furnace at 600 °C for 4 hours. After the smoldering process, it was cooled in a desiccator for 15 minutes to slowly reduce the temperature before weighing. Calculations were made using the following formula:

$$IVDMD = \frac{\text{dry metter of sample} - \text{dry metter of residue}}{\text{dry metter of sample}} \times 100$$

$$IVOMD = \frac{\text{organic metter of sample} - (\text{organic residue weight} - \text{ash weight})}{\text{organic metter of sample}} \times 100$$

Data analysis

The experimental design used in this study was a completely randomized design (CRD) with a factorial arrangement consisting of two factors: factor P, representing the encapsulation method (spray drying, freeze drying, and cell immobilization), and factor M, representing the matrix material (maltodextrin, alginate, and xanthan gum). The study comprised nine treatment combinations, each performed in triplicate. Data on color, aroma, texture, DMD, OMD, IVDMD, IVOMD were analyzed statistically using analysis of variance (ANOVA). Significant differences between treatments were subjected to Duncan's exact test. Data analysis used SPSS software version 25. FTIR data were analyzed using OriginLab 2025 software. TPC and SEM data were analyzed descriptively.

RESULT AND DISCUSSION

Sensory analysis

The quality assessment of symbiotic products was carried out through an sensory analysis involving 25 panelists, with assessment parameters including color, aroma, and texture. The average value of the assessment results is shown in Table 2. The sensory analysis is used to assess the aroma, texture, and color of symbiotic products by utilizing the five senses of the panelists. This method is simple, practical, and fast, although it is subjective because it is strongly influenced by panelist sensitivity (Idris and Aulia 2021; Joris *et al*., 2022). The results of variance analysis showed a significant interaction between the encapsulation method and matrix type ($P<0.05$) on sensory characteristics, which play an important role in consumer acceptance.

Table 2: Sensory analysis results of symbiotic products.

Parameter	Method	Matrix			Average
		M1	M2	M3	
Color	P1	1,20 ± 0,40[a]	1,31 0,46[a]	1,25 ± 0,38[a]	1,25 ± 0,43
	P2	2,40 ± 0,62[b]	2,64 ± 0,48[c]	2,89 ± 0,51[d]	2,64 ± 0,57
	P3	3,48 ± 0,50[f]	3,32 ± 0,57[e]	3,83 ± 0,45[g]	3,54 ± 0,55
	Average	2,36 ± 1,06	2,42 ± 0,97	3,54 ± 0,55	
Aroma	P1	2,47 ± 0,85[cd]	2,61 ± 0,75[d]	2,52 ± 0,78[cd]	2,53 ± 0,86
	P2	2,32 ± 0,54[bc]	2,28 ± 0,62[bc]	2,36 ± 0,64[bc]	2,32 ± 0,68
	P3	2,12 ± 0,99[b]	1,13 ± 0,35[a]	2,08 ± 1,21[b]	1,78 ± 1,18
	Average	2,30 ± 0,98	2,42 ± 0,17	2,32 ± 1,02	
Texture	P1	1,61 ± 0,68[a]	2,00 ± 1,01[b]	2,05 ± 0,96[b]	1,88 ± 0,91
	P2	2,37 ± 0,65[cd]	2,17 ± 0,84[bc]	2,52 ± 0,66[d]	2,36 ± 0,74
	P3	3,76 ± 0,54[e]	3,91 ± 0,44[e]	3,83 ± ± 0,48[e]	3,82 ± 0,49
	Average	2,58 ± 1,09	2,69 ± 1,18	2,80 ± 1,04	

Score: 1 = white; characteristic fermented aroma; very smooth; 2 = yellowish; caramel aroma; smooth; 3 = brown; odorless; slightly rough; 4 = dark brown; unpleasant odor; rough. Notes: P1 = spray drying, P2 = freeze drying, P3 = cell immobilization, M1 = maltodextrin, M2 = alginate, M3 = xanthan gum. abc Different superscripts in the same row and column indicate significant differences ($P < 0.05$).

Table 3: Physicochemical profile of symbiotic products.

Parameter	Method	Matrix			Average	*P-value*
		M1	M2	M3		
PPR (A_{1510}/A_{898})	P1	1.10 ± 0.00	1.09 ± 0.00	1.10 ± 0.00	1.10 ± 0.00[a]	0.14
	P2	1.06 ± 0.04	1.05 ± 0.02	1.07 ± 0.01	1.06 ± 0.02[b]	
	P3	1.03 ± 0.00	1.03 ± 0.00	1.05 ± 0.01	1.03 ± 0.01[c]	
	Average	1.06 ± 0.04	1.05 ± 0.03	1.07 ± 0.02		
IP (A_{1600}/A_{1508})	P1	0.94 ± 0.01	0.94 ± 0.01	0.94 ± 0.01	0.94 ± 0.00[a]	0.01
	P2	0.96 ± 0.02	0.95 ± 0.02	0.93 ± 0.03	0.95 ± 0.02[a]	
	P3	0.85 ± 0.03	0.87 ± 0.02	0.80 ± 0.03	0.84 ± 0.04[b]	
	Average	0.92 ± 0.55[a]	0.92 ± 0.05[a]	0.89 ± 0.07[b]		
HBI (A_{3400}/A_{1320})	P1	0.97 ± 0.01[cd]	0.98 ± 0.00[cd]	0.99 ± 0.02[abc]	0.98 ± 0.01	0.38
	P2	1.02 ± 0.01[a]	1.01 ± 0.01[a]	1.00 ± 0.01[ab]	1.01 ± 0.01	
	P3	0.98 ± 0.01[bc]	0.98 ± 0.02[bc]	0.95 ± 0.02[a]	0.97 ± 0.02	
	Average	0.99 ± 0.02	0.99 ± 0.02	0.98 ± 0.03		
TCI (A_{1372}/A_{2900})	P1	0.94 ± 0.01	0.94 ± 0.00	1.10 ± 0.00	0.94 ± 0.00[b]	0.04
	P2	0.94 ± 0.00	0.94 ± 0.00	0.94 ± 0.00	0.95 ± 0.02[a]	
	P3	0.96 ± 0.00	0.96 ± 0.01	0.94 ± 0.01	0.95 ± 0.01[a]	
	Average	0.95 ± 0.01[a]	0.95 ± 0.01[a]	0.94 ± 0.01[b]		
LOI (A_{1430}/A_{898})	P1	1.02 ± 0.00[a]	1.01 ± 0.00[a]	1.02 ± 0.01[a]	1.02 ± 0.01	0.12
	P2	1.02 ± 0.02[a]	1.01 ± 0.00[a]	1.01 ± 0.00[a]	1.01 ± 0.01	
	P3	0.96 ± 0.01[b]	0.95 ± 0.01[b]	0.95 ± 0.01[c]	0.95 ± 0.01	
	Average	1.00 ± 0.03	0.99 ± 0.03	0.99 ± 0.03		

Notes: L/C (ratio lignin cellulose), CLL (cross-link lignin), HBI (hidrogen bond index), TCI (total crystalline index), dan LOI (lateral order index), P1=spray drying, P2= freeze drying, P3 = cell immobilization , M1= maltodextrin, M2 = alginate, M3 = xanthan gum.

The spray-drying method (P1) produced the brightest color with an average score of 1.25 ± 0.43, close to the white value. This was influenced by the rapid drying process of only a few milliseconds, which reduced color degradation (Sharma *et al*., 2022). In contrast, cell immobilization (P3) produced a dark color (3.54 ± 0.55) due to slow drying at 50°C for 24 hours which triggered browning reactions.

In terms of aroma, spray drying (P1), especially the combination of P1M2, obtained the highest score (2.61 ± 0.75) because high heating produces caramel aroma (Herminiati *et al.,* 2015). Freeze drying (P2) showed a stable score (2.32 ± 0.68) thanks to its ability to retain volatile compounds at low temperatures (Habibi *et al.*, 2019). Meanwhile, the cell immobilization (P3) treatment obtained the lowest aroma score (1.78 ± 1.18). This result indicates that the solid bead structure may have limited the diffusion of volatile compounds, resulting in a lower aroma perception by the panelists. However, since no direct measurement of volatile retention or release (e.g., using GC–MS or headspace analysis) was conducted, this interpretation remains hypothetical. These findings are consistent with previous research on alginate-based encapsulation systems, where the cross-linked Ca^{2+}–alginate network enhanced the retention of volatile compounds by restricting their diffusion. A previous study by Cvitković *et al.* (2025) also reported that increasing the cross-linking density within the alginate matrix significantly improved the retention efficiency of aromatic and bioactive compounds during drying and storage. This mechanism may explain the lower aroma perception observed in the P3 (cell immobilization) treatment, as the release of volatile compounds was likely hindered by the dense gel structure. Nevertheless, further analytical confirmation is required to validate this assumption.

In the texture parameter, cell immobilization (P3) produced a rough texture with a high score (3.82 ± 0.49) due to the formation of beads through the ionic reaction of Ca^{2+}-alginate and oven drying. Spray drying (P1) gave the smoothest texture (1.88 ± 0.91) with uniform and soluble micro-particles, while freeze drying (P2) produced an intermediate texture (2.17-2.52) with a hollow structure due to ice sublimation (Oyinloye and Yoon, 2020).

Scanning electron microscopy (SEM)

SEM works by focusing an electron beam in a vacuum environment using an electromagnetic lens and detecting electrons reflected from the material's surface to form detailed images. This technique is commonly used in materials science to evaluate the physical and surface characteristics of a material (Ural, 2021). SEM enables visualization of micro- and nanoscale details that are not visible under a light microscope, with magnifications exceeding 10,000×. The process uses a high-energy electron beam that is focused to produce images based on the interaction between electrons and the specimen. The images are formed from secondary electron (SE) and backscattered electron (BSE) signals, which provide information about the specimen's topography and surface structure (Mohammed and Abdullah, 2018).

The observations (Figure 2) showed that the morphology of the symbiotic particles was strongly influenced by both the encapsulation method and the type of coating matrix used. In the spray-dried samples (Figure 2A-C), the particles appeared spherical and relatively uniform, with surfaces ranging from smooth to slightly wrinkled. The use of maltodextrin (P1M1, Figure 2A) resulted in smooth and compact surfaces without cracks, indicating its ability to form a stable protective layer during rapid drying. Alginate (P1M2, Figure 2B) produced spherical particles with slightly wrinkled surfaces, while xanthan gum (P1M3, Figure 2C) showed more pronounced surface shrinkage with irregular folds. These surface deformations occurred due to the rapid release of moisture at high temperatures, which caused uneven contraction of the capsule wall, leading to a textured rather than fully collapsed morphology. These findings are consistent with the statement of Bhagwat *et al.* (2020), who reported that spray-dried microcapsules generally exhibit a spherical shape with a deflated yet compact surface, indicating that although the surface may appear wrinkled, the overall structure remains predominantly spherical and the capsule wall is not damaged. Similarly, Shofinita *et al.* (2025) reported that spray-dried microcapsules with compact wall structures can effectively maintain the integrity of probiotic cells. In addition, Zhou *et al.* (2023) found that the use of coating matrices such as whey protein isolate promotes the rapid formation of a stable protective layer under high inlet temperatures.

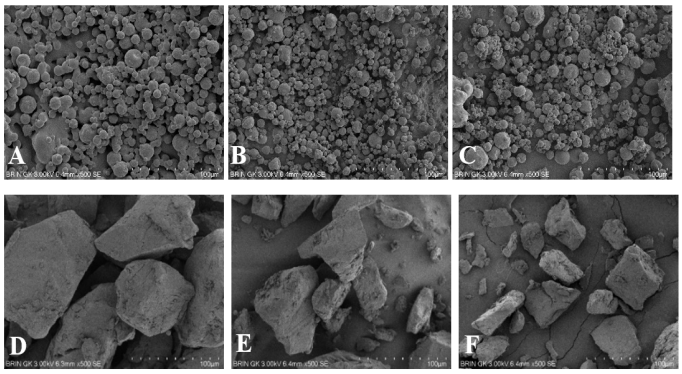

Figure 2: SEM results of product symbiotic.
Notes: (A): P1M1, (B): P1M2, (C): P1M3, (D): P3M1, (E): P3M2, (F): P3M3; P1=spray drying, P3 = cell immobilization, M1= maltodextrin, M2 = alginate, M3 = xanthan gum. Scale = 100µm.

In contrast, particles produced by the cell immobilization method (Figure 2D-F) exhibited irregular, angular, and rough-textured shapes. Maltodextrin (P3M1, Figure 2D) resulted in large, compact aggregates, alginate (P3M2, Figure 2E) produced irregular block-like shapes with hard surfaces due to the formation of a stable three-dimensional gel network through ionic bonding between carboxylate groups and Ca^{2+} ions, and xanthan gum (P3M3, Figure 2F)

showed the roughest and most fractured structures with varied particle sizes. Different coating matrices produced distinct morphological characteristics: Maltodextrin tended to generate smoother and more uniform surfaces, whereas alginate and xanthan gum produced rougher and denser textures depending on their gel-forming properties. Meanwhile, the freeze-drying method (P2) could not be analyzed using SEM because the samples were sticky and hygroscopic, which interfered with the conductive coating process required for SEM observation. This condition is likely caused by residual moisture and sugar content from the MRS-broth culture medium that was not completely sublimated during the lyophilization process. According to Nowak and Jakubczyk (2020), exceeding the glass transition temperature (Tg) during freeze-drying may lead to structural collapse and a reduction in product porosity, which consequently prolongs the secondary drying stage, decreases rehydration capacity, and increases the final moisture content, ultimately reducing product stability. Additionally, as described by Oyinloye and Yoon (2020), many food materials contain amorphous glassy components such as sugars that are thermodynamically unstable; under inadequate drying conditions or excessive temperature, these components may transition into a viscous phase, resulting in stickiness and caking. This phenomenon explains the caramel-like, non-powdery texture observed in P2, indicating incomplete sublimation and structural collapse during the freeze-drying process. To overcome the limitations encountered in this study, future research is recommended to utilize cryo-scanning electron microscopy (cryo-SEM), which enables direct observation of high-moisture or fragile samples without extensive dehydration or metal coating. Conventional SEM techniques are unsuitable for such samples, as water removal under high vacuum conditions often leads to structural deformation or collapse. In contrast, cryo-SEM allows the analysis of the ultrastructure of hydrated samples while preserving their native morphology. Samples are rapidly frozen and maintained at low temperatures within the microscope chamber, ensuring structural stability and minimizing artifacts typically caused by drying or coating processes. This technique is faster and requires simpler preparation compared to conventional SEM methods based on freeze-drying, and has proven highly effective for examining fine morphological features and structural development in hydrated or gel-based systems, while maintaining the integrity of delicate samples (Sriamornsak *et al*., 2008).

Although no morphological images were obtained for P2, the relatively high bacterial viability in this treatment can be explained by the gentle drying process at low temperatures, which minimizes thermal stress and maintains cell membrane integrity. However, compared to the immobilization method (P3), the freeze-dried matrix likely provides lower mechanical protection, as it tends to form a porous and fragile structure that is easily damaged during handling or rehydration. This explanation is in line with reports by Buljeta *et al*. (2022) and Bhagwat *et al*. (2020), which state that although freeze drying can maintain microbial survival, immobilization techniques offer stronger structural encapsulation through the formation of cross-linked gel networks such as calcium alginate matrices.

From an applied perspective, the spherical and smooth particle shape in the spray drying method (P1) facilitates mixing with feed ingredients without causing clumping, resulting in a more even and efficient distribution of probiotics in the ration. This morphology also provides protection against extreme conditions in the stomach and bile, increasing the survival of probiotic cells until they reach the intestine. Arepally *et al*. (2020) reported that probiotic cells encapsulated by spray drying had higher viability than free cells in simulated gastrointestinal fluid, enabling them to interact more optimally with prebiotics (inulin) to improve gut microbiota balance, increase crude fiber digestibility, and enhance nutrient utilization efficiency. Meanwhile, the solid and compact structure of the cell immobilization method (P3) provides better mechanical protection during the digestive process, allowing more probiotic cells to survive until they reach the post-rumen tract. Overall, each method has specific advantages: spray drying excels in industrial efficiency and ease of application, freeze drying provides good thermal protection, while cell immobilization offers the highest mechanical stability and structural protection for probiotics.

Fourier transform infrared spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) is a technique used to determine the molecular structure of organic and inorganic materials without damaging the sample. This technique is commonly applied in the analysis of rocks, coal, minerals, glass, and microfossils. Its working principle involves irradiating the sample with infrared light. When molecules absorb this light, their bonds vibrate either stretching or bending. Each type of bond produces a unique absorption pattern, like a "fingerprint," which can be used to identify specific functional groups such as C–H, O–H, or C=O. Molecules with an uneven charge distribution (dipole moment) can be detected using this technique. FTIR is generally employed in the mid-infrared region, ranging from 4000 to 400 cm^{-1} (Chen *et al*., 2015).

The FTIR spectra presented in Figure 3 demonstrate variations in both the position and intensity of absorption

bands among treatments, reflecting differences in functional group interactions influenced by the encapsulation method and the type of coating matrix used. In general, the P3 (cell immobilization) treatment exhibited a more complex and intense absorption pattern compared to P1 and P2. Distinct absorption bands were observed at approximately 1606–1599 cm^{-1} corresponding to C=C stretching vibrations of aromatic groups, and at 1487–1483 cm^{-1} attributed to C–H deformation in aromatic rings. The increased intensity in these regions indicates stronger lignin–polysaccharide or protein–polysaccharide interactions, suggesting a more organized matrix structure. A dominant band was also detected around 1031–1028 cm^{-1}, representing C–O and C–O–C stretching vibrations of glycosidic bonds in polysaccharides, along with a band near 850–847 cm^{-1} associated with C–H or β-glycosidic linkages. These spectral characteristics indicate that the cell immobilization method promoted the formation of a denser and more stable gel network through cross-linking interactions. In agreement with the findings of Pongjanyakul and Puttipipatkhachorn (2007), after cross-linking with calcium ions, the FTIR spectrum exhibited a clear shift toward higher wavenumbers, accompanied by a decrease in the intensity of the COO^- stretching peaks and a weakened band at 1031 cm^{-1}. These spectral variations indicate the presence of ionic interactions between calcium ions and the carboxyl groups of sodium alginate, as well as partial covalent bonding between calcium and the oxygen atoms within ether linkages.

For the spray-drying method (P1), the main absorption peaks appeared at approximately 1181–1112 cm^{-1}, 1024 cm^{-1}, and 938 cm^{-1}. The region between 1181–1112 cm^{-1} corresponds to C–O and C–O–H stretching vibrations of alcohol and ether groups in polysaccharides, indicating the presence of major functional groups derived from coating materials such as maltodextrin, alginate, and xanthan gum. The peak at 1024 cm^{-1} represents C–O–C glycosidic stretching vibrations typical of inulin and other polysaccharides, while the band at 938 cm^{-1} is associated with C–H stretching of glycosidic linkages. These results confirm that the main polysaccharide structures were preserved despite exposure to high temperatures during spray drying. However, minor intensity variations compared to P2 and P3 suggest slight changes in hydrogen bond orientation or crystallinity due to rapid dehydration during the drying process. In the freeze-drying method (P2), the major absorption bands appeared in the regions of 1031–989 cm^{-1} and 933–847 cm^{-1}, which are characteristic of C–O and C–H stretching vibrations of carbohydrate compounds. Additional small peaks were observed between 1181–1121 cm^{-1} indicating that glycosidic bonds remained intact. The absence of new peaks suggests that the freeze-drying process did not induce significant chemical modifications, thereby preserving the stability of inulin and the coating materials. This result demonstrates that low-temperature drying effectively minimizes molecular degradation.

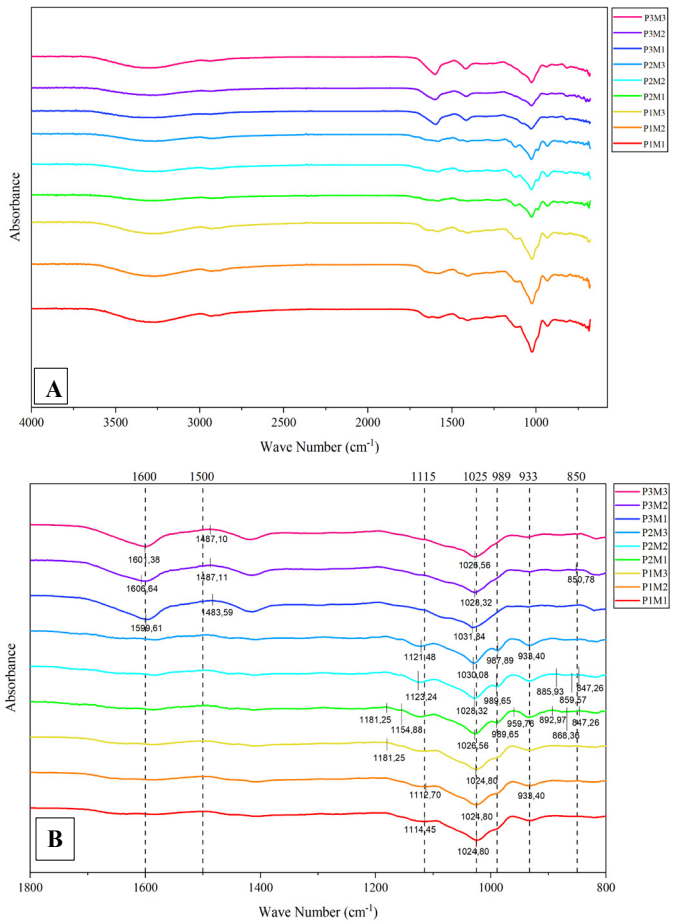

Figure 3: FTIR peak results of symbiotic encapsulant samples.

Notes: (a) The FTIR spectrum showing the overall absorption profile of the samples across the wavenumber range of 4000–600 cm^{-1}, (b) The enlarged FTIR spectrum focusing on the main absorption region between 1800–800 cm^{-1} to highlight specific functional group interactions; P1=spray drying, P2= freeze drying, P3 = cell immobilization, M1= maltodextrin, M2 = alginate, M3 = xanthan gum.

FTIR analysis provided information not only on functional groups but also on structural parameters describing molecular organization within the synbiotic matrix, as presentedin Table 3. The Protein–Polysaccharide Ratio (PPR), Ionic Polysaccharides Index (IP), and Hydrogen Bond Index (HBI) reflect the balance between protein and polysaccharide components, as well as the strength of ionic and hydrogen bonding interactions. Higher PPR values indicate a protein-dominant matrix, while stronger IP and HBI interactions contribute to improved structural integrity, moisture regulation, and protection of B. bifidum cells during drying and storage. The Total Crystallinity

Index (TCI) and Lateral Order Index (LOI) describe the degree of polysaccharide crystallinity and lateral molecular packing, both of which influence matrix compactness and stability. Collectively, these parameters (PPR, IP, HBI, TCI, and LOI) determine the mechanical strength, permeability, and protective capacity of the encapsulation system. More ordered and tightly packed matrices enhance probiotic viability throughout processing but may also reduce porosity and slow probiotic release in the gastrointestinal tract.

FTIR analysis also revealed significant interactions ($P<0.05$) between encapsulation methods and matrix types on the HBI (Hydrogen Bond Intensity) and LOI (Lateral Order Index) parameters. Treatment methods had a significant effect ($P<0.05$) on Protein–Polysaccharide Ratio (PPR), Ionic Polysaccharides Index (IP), and TCI (total crystalline index). P2 produced a simple spectrum, while P1 and P3 exhibited more additional bands due to interactions or cross-linking from the matrix materials. In P1, the high-temperature spray drying process may trigger Maillard reactions between MRS-B components and polysaccharides, while in P3, cell immobilization and drying at 50 °C allowed the formation of more complex cross-links such as ester, carbonyl, and C–O–C groups. This complexity reflects the formation of an amorphous structure with tightly packed interchain polymer bonds.

The density and stability of the gel network in treatment P3 also influence the gradual release of matrix components during degradation and fermentation processes, as reflected in the in vitro dry matter (DM) and organic matter (OM) digestibility patterns, which showed a tendency to increase compared to other treatments, although the difference was not statistically significant. A more stable matrix structure may lead to a more controlled degradation process and a regulated release of active compounds, thereby potentially enhancing the overall effectiveness of the encapsulation system. This observation aligns with previous findings indicating that xanthan gum–based hydrogels with tightly cross-linked structures exhibit high resistance to digestive conditions and facilitate controlled release (Alaoui et al., 2023; Koh *et al*., 2022).

Moreover, studies by Liu *et al*. (2023), Ahmadi *et al*. (2025), and Madybekova *et al*. (2024) also confirmed that FTIR spectral changes can reflect hydrogen bond formation and cross-linking that strengthen encapsulation structures, thereby improving probiotic viability during simulated gastrointestinal transit. Thus, the FTIR spectral changes observed in treatment P3 not only indicate structural modifications at the molecular level but also correlate with improved protection and a more directed degradation mechanism of the matrix, supporting the biological function of the symbiotic encapsulation system.

Viability of encapsulated symbiotics

Probiotic viability refers to the ability of microbial cells to remain alive, active, and capable of reproducing after undergoing processing and storage (FAO/WHO, 2002). A high level of viability indicates that the probiotic maintains its biological activity, which is a key factor determining the effectiveness of a symbiotic product. In animal feed applications, the minimum recommended probiotic population is approximately 10^6–10^7 CFU/g to produce a positive physiological effect in livestock (FAO/WHO, 2001). Values below this range are generally considered insufficient to balance intestinal microflora or improve animal performance. In this study, all treatments met the recommended standard, with viable counts ranging from 10^{12}–10^{14} CFU/g.

Based on the results presented in Table 4, the viability level of the symbiotic product after being re-incubated for 24 hours varied depending on the drying method and the type of coating material used. The viability ranged from 75.59% to 87.90%, with the highest value obtained from the P3M2 treatment (cell immobilization using alginate) at 87.90%, followed by P3M3 (87.30%) and P2M2 (81.52%). Meanwhile, the lowest viability values were found in P2M1 (freeze drying with maltodextrin) at 75.59%, followed by P1M1 (75.94%). These results indicate that the cell immobilization method (P3) provided the most effective protection for probiotic cells compared with spray drying (P1) and freeze drying (P2).

Table 4: Viability level of the symbiotic product after 24 hours (Log CFU/g).

Treatment	Bacterial population (Log CFU g^{-1})	Viability (%)
Before encapsulation	16.71 ± 0.12	
P1M1	12.69 ± 0.09	75.94
P1M2	13.52 ± 0.07	80.88
P1M3	12.75 ± 0.08	76.26
P2M1	12.63 ± 0.05	75.59
P2M2	13.63 ± 0.05	81.57
P2M3	12.92 ± 0.08	77.27
P3M1	13.59 ± 0.11	81.32
P3M2	14.69 ± 0.09	87.90
P3M3	14.59 ± 0.11	87.30

Notes: P1=spray drying, P2= freeze drying, P3 = cell immobilization, M1= maltodextrin, M2 = alginate, M3 = xanthan gum.

The advantages of P3 are most likely due to its process, which does not involve high temperatures or extreme dehydration, thereby preserving cell structure. Additionally, the protective alginate–xanthan gum gel forms a stable barrier that shields bacteria from environmental stress (Grzywaczyk *et al*., 2021). In terms of matrix type, alginate (M2) consistently produced the highest population in all methods. The ability of alginate to form a viscoelastic gel not only protects cells from heat, physical pressure, and extreme pH, but also supports the formation of dense colonies through the quorum sensing mechanism (Huang *et al*., 2023). Conversely, the use of maltodextrin (M1) resulted in the lowest viability values, possibly due to exposure to

high temperatures during the spray drying process and its less effective properties as a barrier against heat and oxygen. Thus, the P3M2 combination can be considered the most effective treatment in maintaining bacterial populations, thereby potentially producing a more stable and beneficial symbiotic product for animal feed.

In vitro DEGRADATION AND DIGESTIBILITY

Based on the data in Table 5, there were no significant differences (P>0.05) in dry matter degradation (DMD), organic matter degradation (OMD), dry matter digestibility (IVDMD), or organic matter digestibility (IVOMD) between encapsulation methods and types of matrices used. These results indicate that synbiotic supplementation did not interfere with rumen fermentation processes or the activity of mature rumen microbiota, whose ecosystem is already well established. In line with the findings of Ahmad *et al.* (2022), the rumen bacterial community in Mongolian cattle reaches stability and maturity with age after weaning, so that changes in the microbial composition in adult animals are only minor. Thus, the stability of the rumen ecosystem in adult dairy cows means that the addition of symbiotics does not show a significant difference in degradation or digestibility.

The stability of these results is a positive indication that encapsulated synbiotics are safe to be used as feed additives, as they do not inhibit rumen fermentation, the central site of fiber digestion in ruminants. Moreover, a tendency toward higher degradation and digestibility values in the cell immobilization treatment (P3), although not statistically significant, suggests a potential benefit that may become more evident at higher supplementation levels or under different physiological conditions of the animals. The absence of significant differences among treatments also reflects the limitations of the in vitro method, which simulates digestion only up to the pepsin–HCl stage representing the true stomach (abomasum), and therefore does not fully capture the digestive activities occurring in the small and large intestines. These sections of the gastrointestinal tract are the primary sites of probiotic and prebiotic action, where microbial colonization, nutrient competition, immune system stimulation, and the production and utilization of various bioactive compounds take place.

The digestibility values obtained in this study were within the normal ranges reported by McDonald et al. (2010), namely 55%–80% for IVDMD and 58%–92% for IVOMD.

Table 5: *In vitro* rumen degradation and digestibility.

Parameter	Method	Matrix			Average	*P-value*
		M1	M2	M3		
DMD	P0	56.30 ± 0.00	56.30 ± 0.00	56.30 ± 0.00	56.30 ± 0.00	0.33
	P1	54.22 ± 3.08	57.27 ± 5.48	55.79 ± 1.61	55.76 ± 3.50	
	P2	57.21 ± 1.90	56.76 ± 0.64	53.18 ± 3.98	55.72 ± 4.07	
	P3	59.40 ± 7.58	54.70 ± 0.53	55.91 ± 1.78	56.67 ± 4.44	
	Average	56.94 ± 4.76	56.25 ± 3.01	54.96 ± 2.68		
OMD	P0	59.88 ± 0.00	59.88 ± 0.00	59.88 ± 0.00	59.88 ± 0.00	0.43
	P1	60.11 ± 1.45	59.60 ± 0.65	57.14 ± 3.49	58.94 ± 2.36	
	P2	56.78 ± 1.24	55.17 ± 0.56	51.06 ± 4.72	54.34 ± 3.54	
	P3	61.83 ± 5.07	58.18 ± 0.46	59.47 ± 1.40	59.83 ± 3.09	
	Average	59.77 ± 3.62	58.94 ± 2.76	58.50 ± 2.33		
IVDMD	P0	66.52 ± 0,00	66.52 ± 0,00	66.52 ± 0,00	66.52 ± 0,00	0.18
	P1	66.04 ± 2.15	65.91 ± 2.74	68.42 ± 1.24	66.78 ± 2.21	
	P2	67.37 ± 1.74	66.04 ± 0.81	65.83 ± 2.27	66.42 ± 1.65	
	P3	67.31 ± 1.85	65.61 ± 2.07	70.31 ± 2.51	67.74 ± 2.78	
	Average	66.91 ± 1.78	65.86 ± 1.77	68.19 ± 2.65		
IVOMD	P0	66.26 ± 0,00	66.26 ± 0,00	66.26 ± 0,00	66.26 ± 0,00	0.30
	P1	64.99 ± 2.83	64.73 ± 3.37	67.63 ± 1.45	65.78 ± 2.70	
	P2	66.84 ± 1.48	65.57 ± 1.02	65.97 ± 1.70	66.51 ± 2.54	
	P3	66.51 ± 2.54	65.04 ± 1.90	69.74 ± 2.17	67.10 ± 2.83	
	Average	66.11 ± 2.21	65.11 ± 2.03	67.63 ± 2.259		

Notes: DMD: dry metter degradation, OMD: organic matter degradation, IVDMD: dry metter digestibility, IVOMD: organic metter digestibility, P0=control without added symbiotic, P1=spray drying, P2= freeze drying, P3 = cell immobilization, M1= maltodextrin, M2 = alginate, M3 = xanthan gum.

This further supports that the lack of significant differences among treatments was not due to impaired digestive function, but rather to the stability of rumen fermentation in adult cattle and the inherent limitations of in vitro methods in representing synbiotic mechanisms throughout the entire gastrointestinal tract. Consequently, encapsulated synbiotics remain promising for providing greater benefits in the post-ruminal phase, particularly in modulating intestinal microbiota, without exerting negative effects on rumen fermentation.

To obtain a more comprehensive understanding of synbiotic effectiveness, further evaluation using multi-stage simulation models that include small and large intestinal conditions, or through in vivo trials, is required. Such approaches would allow a more complete elucidation of how encapsulated synbiotics interact with the digestive environment and contribute to the health and productivity of ruminants.

CONCLUSION

The cell immobilization encapsulation method with a combination of alginate matrix is the most recommended encapsulation method because it is capable of producing microcapsules with a stable gel structure, maintaining the highest bacterial viability, and showing good protected to degradation and rumen digestibility and optimal potential for post-rumen delivery.

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NOVELTY STATEMENT

This study provides a comprehensive comparison of three encapsulation strategies spray drying, freeze drying, and cell immobilization for symbiotic *Bifidobacterium bifidum* and inulin. The findings highlight the superior protective performance of cell immobilization using alginate, resulting in significantly higher bacterial viability and improved functional stability. This research offers new insights into optimizing encapsulation approaches for symbiotic applications in both feed and animal nutrition.

AUTHOR'S CONTRIBUTION

Annisa' Fadhilatul Ummah conducted the experimental work, carried out the laboratory analyses, and prepared the initial manuscript draft. Indah Wijayanti conceptualized and supervised the study, provided methodological and analytical guidance, and critically revised the manuscript. Mulyorini Rahayuningsih contributed to the cell-immobilization encapsulation method, supervised the generated data, and reviewed the manuscript. Roni Ridwan provided guidance and interpretation of the FTIR and SEM data and reviewed the manuscript. All authors reviewed, edited, and approved the final version of the manuscript.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI or AI-assisted technologies were used to generate, analyze, or interpret the research data. Generative AI tools were used only to assist in improving the grammar and readability of the manuscript during the revision stage, and all scientific interpretations and conclusions are entirely the authors' own.

Conflict of interest

The authors have declared no conflict of interest.

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